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Published in:
Toxicon

Link to article, DOI:
[10.1016/j.toxicon.2015.03.001](https://doi.org/10.1016/j.toxicon.2015.03.001)

Publication date:
2015

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Laustsen, A. H., Gutiérrez, J. M., Lohse, B., Rasmussen, A. R., Fernández, J., Milbo, C., & Lomonte, B. (2015). Snake venomics of monocled cobra (*Naja kaouthia*) and investigation of human IgG response against venom toxins. *Toxicon*, 99, 23-35. <https://doi.org/10.1016/j.toxicon.2015.03.001>

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Snake venomomics of monocled cobra (*Naja kaouthia*) and
investigation of human IgG response against venom toxins

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Running title: Proteomics of monocled cobra venom

Keywords: *Naja kaouthia*; monocled cobra; snake venom; proteomics; toxicity; human
IgG response; immunity

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29 **Highlights**

30

- 31 • The venom proteome of the monocled cobra, *Naja kaouthia*, is presented
- 32 • Most abundant venom components are three-finger toxins (neurotoxins and
- 33 cytotoxins) and phospholipases A₂
- 34 • Toxicity (LD₅₀) screening identified three-finger toxins as the most medically
- 35 relevant of this venom
- 36 • IgG response from a self-immunized human was significant against several toxins,
- 37 although weak against those with higher toxicity

38

Abstract

The venom proteome of the monocled cobra, *Naja kaouthia*, from Thailand, was characterized by RP-HPLC, SDS-PAGE, and MALDI-TOF-TOF analyses, yielding 38 different proteins that were either identified or assigned to families. Estimation of relative protein abundances revealed that venom is dominated by three-finger toxins (77.5%; including 24.3% cytotoxins and 53.2% neurotoxins) and phospholipases A₂ (13.5%). It also contains lower proportions of components belonging to nerve growth factor, ohanin/vespryn, cysteine-rich secretory protein, C-type lectin/lectin-like, nucleotidase, phosphodiesterase, metalloproteinase, L-amino acid oxidase, cobra venom factor, and cytidyltransferase protein families. Small amounts of three nucleosides were also evidenced: adenosine, guanosine, and inosine. The most relevant lethal components, categorized by means of a 'toxicity score', were α -neurotoxins, followed by cytotoxins/cardiotoxins. IgGs isolated from a person who had repeatedly self-immunized with a variety of snake venoms were immunoprofiled by ELISA against all venom fractions. Stronger responses against larger toxins, but lower against the most critical α -neurotoxins were obtained. As expected, no neutralization potential against *N. kaouthia* venom was therefore detected. Combined, our results display a high level of venom complexity, unveil the most relevant toxins to be neutralized, and provide prospects of discovering human IgGs with toxin neutralizing abilities through use of phage display screening.

(199 words)

1. Introduction

Snakebite is a serious medical condition affecting a large number of people worldwide; especially in impoverished rural areas of Asia, Africa and Latin America (Gutiérrez et al., 2006; Warrell, 2010a). Asia is the continent where the majority of these bites take place, and also where most deaths occur (Alirol et al., 2010; Chippaux, 1998; Kasturiratne et al., 2008). Currently there are 29 recognized extant species of terrestrial cobras assigned to the genus *Naja* (Uetz and Hošek, 2015). Of these, 11 species are found in Asia, and 18 inhabit Africa (Uetz and Hošek, 2015; Wallach et al., 2009). Among the cobras, *Naja kaouthia* (monocled cobra) is widespread in southern Asia and responsible for a significant part of the bites recorded (Viravan et al., 1986; Warrell, 1995; Kulkaew et al., 2009). A study in Bangladesh discovered records of 764 snakebites during 1988-1989, of which 34% of the 168 deaths (22% case fatality) were attributed to the cobras *Naja naja* and *N. kaouthia* (Warrell, 2010b). *N. kaouthia* occurs from north-eastern India, Bangladesh and Bhutan, across southern China, southward to northern Peninsular Malaysia (Prakash et al., 2012; Wüster, 1996) and is common in most of its range (Stuart and Wogan, 2012). *N. kaouthia* is adapted to a broad range of habitats, including agricultural land, human settlements, and bigger cities. However, its natural habitat includes paddy fields, swamps, mangroves, grasslands, scrublands and forest (Stuart and Wogan, 2012). The diet of *N. kaouthia* covers a broad range of animals from frogs, snakes, small birds to mammals (Cox et al., 1998), and even fish (Kyi and Zug, 2003). *N. kaouthia* is assessed as Least Concern at IUCN Red List of Threatened Species because of its large distribution, tolerance of a broad range of modified habitats, and is reported to be in abundance (Stuart and Wogan, 2012). In Thailand it has been reported that many cases of hospitalization due to snakebite

were caused by *N. kaouthia* (Kulkeaw et al., 2009). Human envenomings by *N. kaouthia* are predominantly characterized by neuromuscular paralysis which in severe cases ends up in respiratory paralysis, and by local tissue damage, i.e. swelling, necrosis and blistering (Warrell, 1995; Wongtongkam et al., 2005).

Enzymatic activities and toxicity of the venom of *N. kaouthia* have previously been studied (Pakmanee et al., 1998; Mukherjee and Maity, 2001; Das et al., 2013). Furthermore, a qualitative proteome of the venom has been reported showing the presence of 61 proteins belonging to 12 protein families (Kulkaew et al., 2007). The most prominent component is the long α -neurotoxin α -cobratoxin (Richard et al., 2013), constituting about 25% of the venom (Kulkaew et al., 2009) and belonging to the three-finger toxin family, of which *N. kaouthia* venom has several other members (Kulkaew et al., 2007). Other proteins that have been studied include phospholipases A₂ (PLA₂s) (Joubert and Taljaard, 1980a; Reali et al., 2003; Doley and Mukherjee, 2003), L-amino acid oxidases (Tan and Swaminathan, 1992; Sakurai et al., 2001), cardiotoxins (Joubert and Taljaard, 1980b; Fletcher et al., 1991; Debnath et al., 2010; Jamunaa et al., 2012), and nerve growth factors (Kukhtina et al., 2001). However, a proteomic analysis of this venom that integrates an estimation of relative protein abundances together with a detailed screening of the toxicity of its various components, is pending. An integrated functional and proteomic characterization of *N. kaouthia* venom is relevant not only for a deeper understanding of the composition of the venom and its relationships to toxicity, but also to establish a basic platform for antivenomic analyses.

Developing more potent antivenoms with better safety profiles is of medical relevance. Besides the traditional animal-derived antivenoms generated by immunization of horses with pools of snake venoms (Gutiérrez et al., 2011), various alternative approaches

have been pursued at the experimental level in order to generate more specific antibody-based therapies aimed at neutralizing the most important toxins in the venom. One approach has been to raise murine antibodies specific towards relevant *N. kaouthia* toxins (Charpentier et al., 1990; Masathien et al., 1994). Another approach for identifying potent antitoxins is phage display screening (Roncolato et al., 2015). By using this methodology, inhibitors against *N. kaouthia* toxins have been identified for both PLA₂s (Chavanayarn et al., 2012) and α -cobratoxin (Stewart et al., 2007; Kulkaew et al., 2009; Richard et al., 2013). These studies discovered inhibitors through screening of phage display libraries based on heavy chain fragments obtained from llama. Although promising, it could, however, be argued that llama-derived inhibitors, despite being much smaller than whole IgG antibodies and therefore likely to be less immunogenic, are still non-human. An alternative to overcome this problem is based on modern antibody humanization procedures (Safdari et al., 2013). In another study, inhibitors based on human single chain Fv (scFv) fragments from non-immunized donors were developed (Kulkaew et al., 2009). These scFv fragments were not effective in rescuing mice injected with venom, which is likely to be due to naïve origin of the gene library for these fragments, since none of the human donors had been exposed to snake venom. Thus, the search for novel approaches to generate human antibodies against cobra venom toxins remains a challenge.

With this study, we report for the first time a venomics-based quantitative estimation of the proteome and a full protein lethality profile for *N. kaouthia* venom, in order to lay the foundation for developing a recombinant antivenom by identifying the most relevant toxins present in this venom. Furthermore, we uncover the presence of human IgG antibodies specific to *N. kaouthia* venom in the serum obtained from a unique individual exposed to low doses of a wide variety of snake venoms over a period of more than 25

years. The binding ability of his antibodies, as well as of those from a person bitten twice by the Southern Burrowing Asp, *Atractaspis bibronii*, was tested against the different venom fractions from *N. kaouthia*, in comparison to a healthy volunteer unexposed to snake venoms. Finally, the neutralizing ability against *N. kaouthia* venom of the purified human IgG antibodies was evaluated in a mouse model.

2. Materials and Methods

2.1 Snake venom

Naja kaouthia venom was obtained from Latoxan SAS, Valence, France. The venom is a pool collected from several specimens, originally from Thailand.

2.2 Venom separation by reverse-phase HPLC and SDS-PAGE

Following the ‘snake venomomics’ analytical strategy (Calvete, 2011), crude venom was fractionated by a combination of RP-HPLC and SDS-PAGE separation steps. Venom (2 mg) was dissolved in 200 μ L of water containing 0.1% trifluoroacetic acid (TFA; solution A) and separated by RP-HPLC (Agilent 1200) on a C₁₈ column (250 x 4.6 mm, 5 μ m particle; Teknokroma). Elution was carried out at 1 mL/min by applying a gradient towards solution B (acetonitrile, containing 0.1% TFA): 0% B for 5 min, 0–15% B over 10 min, 15–45% B over 60 min, 45–70% B over 10 min, and 70% B over 9 min, as previously described (Lomonte et al., 2014a). Manually collected fractions were dried in a vacuum centrifuge, redissolved in water, reduced with 5% β -mercaptoethanol at 100 °C for 5 min, and further separated by SDS-PAGE in 12% gels (Bio-Rad). Proteins were stained with colloidal Coomassie blue G-250, and gel images were acquired on a ChemiDoc[®] recorder using ImageLab[®] software (Bio-Rad).

2.3 Protein identification by tandem mass spectrometry of tryptic peptides

Protein bands were excised from the polyacrylamide gels and subjected to reduction (10 mM dithiothreitol), alkylation (50 mM iodoacetamide), and overnight in-gel digestion with sequencing grade trypsin (Sigma), in 50 mM ammonium bicarbonate at 37°C. The resulting tryptic peptides were extracted with 50% acetonitrile containing 1% TFA, and analyzed by MALDI-TOF-TOF on an AB4800-Plus Proteomics Analyzer (Applied Biosystems). Peptides were mixed with an equal volume of saturated α -cyano-hydroxycinnamic acid (in 50% acetonitrile, 0.1% TFA), and spotted (1 μ L) onto an Opti-TOF 384-well plate, dried, and analyzed in positive reflector mode. TOF spectra were acquired using 1500 shots and a laser intensity of 3000. The ten most intense precursor ions were automatically selected and their TOF/TOF fragmentation spectra were acquired using 500 shots at a laser intensity of 3900. External calibration in each run was performed with CalMix[®] standards (ABSciex) spotted onto the same plate. For protein identification, resulting spectra were searched against the UniProt/SwissProt database using ProteinPilot[®] v.4 and the Paragon[®] algorithm (ABSciex) at $\geq 95\%$ confidence, or manually interpreted. Few peptide sequences with lower confidence scores were manually searched using BLAST (<http://blast.ncbi.nlm.nih.gov>) for protein similarity and protein family assignment.

RP-HPLC fractions corresponding to small molecules, eluting in the initial peaks of the chromatogram, were analyzed by ESI-MS/MS on a Q-Trap[®] 3200 instrument (Applied Biosystems). Samples (10 μ L) were loaded into metal-coated capillary tips (Proxeon) and directly infused into a nano-ESI source (Protana) operated at 1300 V. Spectra were acquired in positive Enhanced Resolution mode. nESI-MS performed in this instrument was also used to determine the isotope-averaged mass of intact proteins in selected peaks

from the RP-HPLC separation. For this purpose, mass spectra were acquired in Enhanced Multicharge mode in the m/z range 700-1700, and deconvoluted with the aid of the Analyst[®] v.1.5 software (ABSciex).

2.4 Relative protein abundance estimations

The relative abundances of the venom proteins identified were estimated by integrating the areas of their chromatographic peaks at 215 nm, using the ChemStation[®] software (Agilent), which correlates with peptide bond abundance (Calvete, 2011). For HPLC peaks containing several electrophoretic bands, their percentage distributions were assigned by densitometry, using ImageLab[®] (Bio-Rad).

2.5 Nucleoside and FAD analysis

The presence of selected nucleosides (adenosine, inosine, guanosine), and flavine adenine dinucleotide (FAD) was determined by spiking a sample of 1 mg of venom with 10 µg of each nucleoside or FAD, respectively, and separating it by reverse-phase HPLC as described in section 2.2. If the nucleoside or FAD peak coincided with a peak already present in a crude venom sample (as judged by the increment in the height of the peak), and if this venom peak showed an ESI-MS spectrum similar to that of the nucleoside or FAD, the identity of venom component was judged to be the same as the nucleoside or FAD. Further confirmation of the molecular identities of the nucleosides was obtained by acquiring their collision-induced dissociation MS/MS spectra in positive mode, using the Enhanced Product Ion tool of the Analyst v1.5 in the QTrap 3200 mass spectrometer as described (Laustsen et al., 2015). The abundance for nucleosides given in Table 1 is only indicative of an approximate value, due to differences in absorbance at 215 nm between

nucleosides and proteins.

2.6 In vitro enzymatic activities

2.6.1. Phospholipase A₂ activity

PLA₂ activity was assayed on the monodisperse synthetic chromogenic substrate 4-nitro-3-octanoyloxybenzoic acid (NOBA) (Holzer and Mackessy, 1996). Twenty-five µL containing various amounts of venom were mixed with 200 µL of 10 mM Tris, 10 mM CaCl₂, 0.1 M NaCl, pH 8.0, and 25 µL of NOBA to achieve a final substrate concentration of 0.32 mM. Plates were incubated at 37 °C for 60 min, and absorbances were recorded at 405 nm in a microplate reader. Samples were assayed in triplicates.

2.6.3 Proteinase activity

Variable amounts of venom (10 to 40 µg) were added to 100 µL of azocasein (10 mg/mL in 50 mM Tris–HCl, 0.15 M NaCl, 5 mM CaCl₂ buffer, pH 8.0), and incubated for 90 min at 37 °C. The reaction was stopped by addition of 200 µL of 5% trichloroacetic acid, and after centrifugation (5 min, 13,000 rpm), 150 µL of supernatants were mixed with 100 µL of 0.5 M NaOH, and absorbances were recorded at 450 nm. The absorbance of azocasein incubated with distilled water alone was used as a blank, being subtracted from all readings (Wang et al. 2004). Samples were assayed in triplicates.

2.7 Toxicological profiling

2.7.1 Animals

In vivo assays were conducted in CD-1 mice, supplied by Instituto Clodomiro Picado, following protocols approved by the Institutional Committee for the Use and Care

of Animals (CICUA), University of Costa Rica. Mice were housed in cages for groups of 4–8, and were provided food and water *ad libitum*.

2.7.2 Toxicity of crude venom and isolated toxins

The lethality of the whole venom and fractions or isolated toxins was tested by intravenous (i.v.) injection in groups of four mice (18–20 g body weight). Various amounts of venom or fractions/toxins were dissolved in phosphate-buffered saline (PBS; 0.12 M NaCl, 0.04 M sodium phosphate buffer, pH 7.2), and injected in the caudal vein, using an injection volume of 100 μ L. Then, deaths occurring within 24 hr were recorded. Median lethal dose (LD₅₀) was calculated by probits (Finney, 1971), using the BioStat[®] software (AnalySoft).

The toxicity of venom fractions was initially screened by selecting a dose based on fraction abundance. The dose was selected to assess whether the fraction would score above or below 5 according to the Toxicity Score defined by Laustsen et al. (2015), which represents the ratio of protein fraction abundance (%) in the venom divided by its estimated LD₅₀ in CD-1 mice by i.v. injection. Fractions that were not lethal at this dose (yielding a Toxicity Score < 5) were considered as having insignificant toxicity. All fractions that were lethal at this level, and some fractions killing mice at a Toxicity Score level between 1-5 were further evaluated, and precise LD₅₀ values were further determined for them. Groups of 3-5 mice per dose were used in order to minimize the use of experimental animals.

2.8 Preparation of human IgGs and human serum

2.8.1 Donor 1

This donor has over a period of 25 year injected himself with sub-lethal amounts of venom from the following snakes: *Viridovipera* (formerly *Trimeresurus*) *stejnegeri*, *Trimeresurus* *nebularis*, *T. macrops*, *T. popeorum*, *T. trigonocephalus*, *T. albolabris*, *T. venustus*, *Bothriechis schlegelii*, *Crotalus enyo enyo*, *Naja kaouthia*, *N. naja*, *N. siamensis*, *Agkistrodon contortrix*, *A. bilineatus*, *Bothrops asper*, *Crotalus oreganus*, *Crotalus oreganus helleri*, *Cerastes cerastes*, and *Micrurus fulvius*. In the last 5 years, this individual has been injecting himself with venom from *Naja kaouthia* on an average of every 2 weeks. Brian Lohse has a written consent from the Danish scientific ethical council (H-3-2013-FSP60) to use the blood sample from this donor according to the law of the ethical committee § 2.1. After having provided written and oral information about the project to this donor, written consent was given for obtaining blood samples and for performing the experiments included in the present work. The donor requested the inclusion of his name to be acknowledged in publications.

2.8.2 Donor 2

Donor 2 is an entomologist, who was unfortunate to be bitten twice by the snake *Atractaspis bibronii* (Southern Burrowing Asp) during fieldwork in August 2013. After having provided written and oral information about the project to this donor, written consent was given for obtaining blood samples and for performing the experiments here included. The donor requested the inclusion of his name to be acknowledged in publications.

2.8.3 Blood sampling for obtaining IgGs

200 ml of blood was sampled from each donor in Vacuette[®] tubes of 4 mL, containing Z Serum Clot Activator (Greiner bio-one). Every tube was gently tilted up and down ten times as soon as it was filled with blood to ensure thorough mixing with the coagulation factor. The tubes were kept at room temperature until full coagulation (30 min). Then, tubes were centrifuged at 3000 g for 10 min, and the serum was transferred to cryotubes and stored at -20 °C.

2.8.4 Protein A purification of IgG antibodies

After filtration of serum through a 0.45 µm membrane, the procedure accompanying the Protein A Antibody Purification Kit (PURE1A, Sigma-Aldrich) was followed in order to obtain human IgG antibodies in solution at physiological pH. IgG antibodies were stored at 4 °C.

2.8.5 Preparation of human serum from healthy volunteer

Blood was obtained from a healthy donor to serve as a negative control. After clotting, serum was separated by centrifugation, and stored at – 20 °C.

2.9 Immunoreactivity of human IgGs against venom fractions by ELISA

Wells in MaxiSorp plates (NUNC, Roskilde, Denmark) were coated overnight with 0.6 µg of each HPLC venom fraction dissolved in 100 µL PBS. Then, wells were blocked by adding 100 µL PBS containing 2% (w:v) bovine serum albumin (BSA, Sigma) and leaving the plates on a mixer at room temperature for 1 h. Plates were then washed five times with PBS. A dilution of each human IgG pool in PBS + 2% BSA was prepared such that the concentration of IgG proteins was 86 µg/mL (as measured by their absorbance at

280 nm on a NanoDrop[®] 2000c instrument, Thermo Scientific). Then, 100 µL of these IgG solutions were added to the wells, after which the plates were incubated for 2 h, followed by five additional washings with PBS. Then, 100 µL of a 1:2000 dilution of affinity-purified goat anti-human IgG (gamma-chain specific) antibodies conjugated to alkaline phosphatase (Cappel, Organon Teknika) in PBS + 2% BSA was added to each well. The plates were incubated for 2 h, and then washed five times with FALC buffer (0.05 M Tris, 0.15 M NaCl, 20 µM ZnCl₂, 1 mM MgCl₂, pH 7.4). Color development was achieved by the addition of 100 µL *p*-nitrophenyl phosphate (1 mg/mL in 9.7% v/v diethanolamine buffer, pH 9.8). The absorbances at 405 nm were recorded (Multiskan FC, Thermo Scientific) at several time intervals.

2.10 Neutralization studies with human IgGs

After purification according to section 2.8.4, the human IgGs were kept refrigerated. Six months later, 3 mL of IgGs were dialyzed against 2 L of PBS overnight and lyophilized. The IgGs were reconstituted in 500 µL PBS and the protein concentration was measured on NanoDrop to be 14 mg/mL. IgG was mixed with venom at a ratio of 78 mg IgG/mg venom and incubated at 37 °C for 30 min. Aliquots of 120 µL of this mixture, containing 4 LD₅₀s of venom, were then injected i.v. into a group of four mice (18-20 g) and deaths occurring within 24 hr were recorded.

3.0 Results and Discussion

3.1 Venomics

A proteomic characterization of *N. kaouthia* venom coupled with an estimation of its protein relative abundances was, with this study, carried out for the first time. Twenty-

eight peaks from the venom were resolved by RP-HPLC. The first three fractions eluting from the column were non-peptidic, while the remaining 25 peaks were further resolved into 50 protein bands by SDS-PAGE separation (Fig.1). In-gel digestion and MALDI-TOF-TOF analysis of these yielded a total of 38 identified proteins, while only 4 remained unknown. Altogether, the unidentified venom components represent less than 1% of the total venom protein content, while the rest were either identified or assigned to protein families (Table 1). A previous qualitative proteomic profile was reported for this venom, describing matches to 61 orthologous proteins classified into 12 groups (Kulkaew et al., 2007). Of these, oxoglutarate dehydrogenase complex was identified on the basis of a single peptide that matched such enzyme from a prokaryote (*Burkholderia cenocepacia*), while serum albumin likely corresponds to contaminating traces derived from slight trauma that may occur during venom collection (see for example Lomonte et al., 2014b). In addition, the previous study considered cardiotoxins and cytotoxins as two different protein groups, in spite of these two names being used to refer to the same type of toxins, structurally classified within the three-finger toxin family (Kini and Doley, 2010). Our results are concordant with the remaining eight protein families detected by Kulkaew et al. (2007), but further expand the proteomic profile of *N. kaouthia* venom with the addition of members belonging to the nerve growth factor, ohanin-like/vespryn, C-type lectin/lectin-like, nucleotidase, phosphodiesterase, L-amino acid oxidase, and cytidyltransferase protein families (Table 1 and Fig.2), and moreover, providing a quantitative estimation of their relative abundances in the venom.

Peaks 1 to 3 were analyzed by direct infusion using nESI-MS/MS, since they did not show proteins by gel electrophoresis. Peaks 2 and 3 had molecular masses of 268 Da and 283 Da, respectively, and the nucleoside analysis by HPLC (Fig.3) revealed their

identities as adenosine and guanosine, respectively. Also, a trace of inosine between peaks 2 and 3 was detected by the nucleoside analysis by HPLC (Fig.3).

All peptidic peaks were tested *in vivo* for acute toxicity, except for peaks 23 and 26, which yielded very low amounts of proteins (Table 2), and peaks 24, 27, and 28, which correspond to metalloproteinases, enzymes known to be inactivated by the RP-HPLC separation procedure. In Fig.2, the overall protein composition of the venom of *N. kaouthia*, expressed as percentage of total protein and nucleoside content, is represented. Two main families predominate in the venom: Three-finger toxins (3FTx; 77.5%, of which 24.3% are cytotoxins, the rest being neurotoxins) and PLA₂s (PLA₂; 13.5%). In partial agreement with previous findings, the major single component of the three-finger toxins was determined to be alpha-elapitoxin-Nk2a (α -cobratoxin), a long-chain neurotoxin. However, in our results, this toxin represented 32.3% of the whole venom, which is significantly higher than the reported values of 23-25% (Kulkaew et al., 2009). This increase might reflect either geographical variations in venom composition or differences in methodology. As other α -neurotoxins, alpha-elapitoxin-Nk2a is able to bind to nicotinic receptors at the motor end-plate of muscle fibers, thus generating a flaccid paralysis leading to respiratory failure and death (Ultsintong et al., 2009; Alkondon et al., 1990). This type of neurotoxin is responsible for the life-threatening neuromuscular paralysis in human victims of neurotoxic cobra bites (Warrell, 1995).

An interesting finding, discovered by the lack of high acute toxicity in the rodent model (Table 2), and reinforced by the difference in human IgG binding in the ELISA assays (Fig.6) was that the protein identified in peak 10 as alpha-elapitoxin-Nk2a appears to correspond to a closely related homolog. In order to validate this, both peak 10 and peak 6

(known to contain alpha-elapitoxin-Nk2a) were analyzed by nESI-MS/MS (Fig.5), according to which their masses were 7619 and 7826 Da, respectively (alpha-elapitoxin-Nk2a has a reported mass of 7831 Da according to: <http://www.uniprot.org/uniprot/P01391>). Thus, peak 10 should be a homolog of alpha-elapitoxin-Nk2a sharing peptide sequences (Table 1), but different by 207 Da in total mass. Since the toxicity of this protein is markedly lower in comparison to alpha-elapitoxin-Nk2a, further studies to determine its complete amino acid sequence and bioactivities could be of interest, as it represents nearly 10% of the venom proteome.

In addition to α -neurotoxins, other representatives of the three-finger toxin family detected in *N. kaouthia* venom proteome are the so-called 'cytotoxins' (Table 1). These toxins are known to interact with and disrupt the integrity of plasma membrane of various cell types, thus inducing irreversible cell damage (Konshina et al., 2012). These cytotoxins are responsible for the local necrotic effect characteristic of envenomings by *N. kaouthia* (Warrell, 1995). Also, PLA₂ is commonly an abundant component in the majority of elapid venom proteomes characterized so far, and it was detected in our proteomic analysis in *N. kaouthia* venom. PLA₂ activity was confirmed for *N. kaouthia* venom *in vitro*, although it was significantly lower than for *M. nigrocinctus*, a Central American elapid species (Fig.4A). Elapid PLA₂s have been shown to exert a number of toxicological effects, such as myonecrosis (Bhat and Gowda, 1989; Harris, 1991), anticoagulation (Kini, 2005; Doley and Mukherjee, 2003), inhibition of platelet aggregation (Clemetson et al., 2007), and edema (Wang and Teng, 1990). In the case of *N. kaouthia* venom, PLA₂s might contribute to the local inflammatory and necrotizing effects observed in human patients. Furthermore, cardiotoxins and PLA₂s act synergistically to induce cell membrane damage (Harvey et al., 1983), thus potentiating the tissue damaging effect of the venom. Altogether, a high

proportion of the components of *N. kaouthia* venom belong to these two toxin families, i.e. three-finger toxins and PLA₂s. This further supports the concept that high diversity within just a few toxin families may be a general theme within elapid venoms (Calvete, 2013; Laustsen et al., 2015). Such idea is supported also by findings of Vonk et al. (2013) on the genome of the elapid *Ophiophagus hannah* (king cobra), in which the three-finger toxins family was found to be highly diversified.

In amounts each representing <2% of the total venom content, the venom also contains members of the nerve growth factor (NGF), Olanin/vespryn (OHA), Cysteine-rich secretory protein (CRISP), C-type lectin/lectin-like (CTL), nucleotidase (NUCL), phosphodiesterase (PDE), metalloproteinase (MP), L-amino oxidase (LAO), Cobra venom factor (CVF), and Cytidyltransferase (CTT) families. The low content of proteinases is further supported by the negligible proteinase activity of the venom upon a general substrate such as azocasein (Fig.4B). Also, regarding non-peptidic material, small amounts were identified to be adenosine, inosine, and guanosine, which have also been detected in other snake venoms (Aird, 2002; Laustsen et al., 2015).

The results of toxicity testing of the fractions are summarized in Table 2. Unsurprisingly, the lethality of the venom stems from the presence of potent α -neurotoxins and cytotoxins, with PLA₂s playing a secondary role leading to local tissue damage at the bite wound. A Toxicity Score is shown in Table 2, which takes into account both potency and abundance. Toxins with a higher score, as estimated in mice, are expected to be of higher medical relevance. These are abundant and/or very potent, while toxins with a low score are less medically relevant. Based on this score, it is clear that alpha-elapitoxin-Nk2a is by far the toxin that is most clinically relevant to neutralize, with a value of 326. Some toxins have very low LD₅₀s (particularly fractions 4 and 5), but due to their lower

abundance their Toxicity Scores are much lower. Others have intermediate LD₅₀ potencies, but become relevant due to their high abundance (e.g. fraction 16). The cytotoxins present in the venom act by a different mechanism than the α -neurotoxins, providing *N. kaouthia* venom with a dual strategy for prey subduction. It is interesting to note that the accumulated Toxicity Scores for all toxins amount to the same value as the Toxicity Score for the whole venom. This is not the case for the venom from the related elapid, *Dendroaspis polylepis*, which shows evidence of synergism, and where the accumulated Toxicity Scores is considerably lower than the Toxicity Score of whole venom (indicating synergism between the toxins and not simply additive effect) (Laustsen et al., 2015). One interpretation of this finding could be that *N. kaouthia* venom does not exert significant synergism, but instead relies on two complementary strategies for acute toxicity.

In terms of therapeutic targets, based on their Toxicity Score, the most relevant toxins in *N. kaouthia* venom that should be neutralized by an effective antivenom are (in order of importance): alpha-elapitoxin-Nk2a (P01391), cobrotoxin-c (P59276) and cobrotoxin-b (P59275), and the homolog of three-finger toxin from *Naja atra* (E2IU03), while the cytotoxins are of less importance in terms of overall lethality, although they are likely to play a relevant role in local necrosis, and hence should be neutralized by antivenoms. In addition, neutralization of PLA₂s should also be considered a priority in order to limit local tissue damage from envenomings. Thus, antivenomic studies with *N. kaouthia* venom should establish whether antivenoms are able to bind these toxins.

3.2 Immunoreactivity of human IgGs against venom fractions

The isolated human IgGs from the unique individual who had self-injected small amounts of venom from various different snakes, including *N. kaouthia*, showed significant

binding ability to isolated venom fractions, clearly above the background established by serum of a healthy donor, or by IgGs from the donor bitten twice by a different snake species (Fig.6). It is evident that the antibody response of the individual exposed to *N. kaouthia* venom was higher against larger toxins present in later eluting peaks, while the response was limited against the most important α -neurotoxins (peaks 4, 5, and 6), which could be due to the low immunogenicity of small toxins (Fernández et al., 2011). Therefore, it was not surprising that IgGs were unable to neutralize the lethality of whole venom when mice were challenged with 4 LD₅₀s mixed with IgGs at a level of 0.013 mg toxin per mg of IgGs. Nevertheless, it could be particularly interesting to further investigate these antibodies via phage display screening. In llamas, it has been shown that using naïve libraries for phage display screening did not yield high affinity inhibitors to snake toxins, while excellent inhibitors of alpha-elapitoxin-Nk2a were discovered this way, when a library from an immunized llama was used (Richard et al., 2013). Kulkeaw et al. (2009) were the first to report drug discovery effort using a human phage display library. However, they similarly did not find strong inhibitors of snake toxins, most likely given the fact that their library was naïve. Therefore, using the antibody genes from the unique donor to construct a phage display library, and subsequently screening this library, has the potential to yield toxin inhibitors of therapeutic value. Further studies of the donor's immune response against toxins from other snake species are warranted and may help direct phage display screening efforts towards the generation of new antibodies of human origin.

4.0 Concluding remarks and outlook

Differing from previous proteomic studies on *N. kaouthia* venom, the present work combined the cataloguing of venom proteins with an estimation of their relative

abundances, together with an assessment of their lethal toxicity for mice. This approach uncovered that the most abundant group of proteins was three-finger toxins, followed by PLA₂s. Based on an evaluation of the accumulation of Toxicity Scores and knowledge about toxin functions, the venom seems to have a dual strategy for prey subduction elicited by α -neurotoxins and cytotoxins, between which pharmacological interaction is speculated only to be of additive value rather than synergism.

ELISA immunoprofiling of human IgGs isolated from a human donor, who had repeatedly injected himself with snake venom from various different species, revealed presence of antibodies with high binding ability to several of the isolated venom fractions, although not against the medically most relevant toxins of *N. kaouthia*. Still, the presence of these antibodies opens the prospect of discovering inhibitors of therapeutic value.

Acknowledgments

The authors thank Steve Ludwin and Thomas Pape for donation of blood samples for this study. We thank Dr. Steven D. Aird (Okinawa Institute of Science and Technology, Japan) for fruitful discussions about nucleosides present in snake venom. We further thank Jens Kringelum (Technical University of Denmark), Mikael Engmark (Technical University of Denmark), and Alexandra Bak Jakobsen (Denmark) for fruitful scientific discussions. We thank the Department of Drug Design and Pharmacology, University of Copenhagen, and Instituto Clodomiro Picado, Universidad de Costa Rica, for supporting the research. Finally, we are grateful to the following foundations for financial support: Drug Research Academy (University of Copenhagen), Dansk Tennis Fond Oticon Fonden, Knud Højgaards Fond, Rudolph Als Fondet, Henry Shaws Legat, Læge Johannes Nicolai Krigsgaard of Hustru Else Krogsgaards Mindelegat for Medicinsk Forskning og

489 Medicinske Studenter ved Københavns Universitet, Lundbeckfonden, Torben og Alice
490 Frimodts Fond, Frants Allings Legat, Christian og Ottilia Brorsons Rejselegat for Yngre
491 Videnskabsmænd og -kvinder, and Fonden for Lægevidenskabens Fremme.

492

493 **Conflicts of interest statement**

494 The authors declare that there are no conflicts of interest related to this study.

495 Sources that provided financial support were not involved in the collection, analysis, or
496 interpretation of data, nor in writing the report and submitting it for publication.

497

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- 677

Figure legends

Figure 1: Separation of *N. kaouthia* (A) venom proteins by RP-HPLC (C), followed by SDS-PAGE (B). Two mg of venom were fractionated on a C₁₈ column and eluted with an acetonitrile gradient (dashed line), as described in Methods. Fractions were further separated by SDS-PAGE under reducing conditions. Molecular weight markers (Mw) are indicated in kDa. Coomassie-stained bands were excised, in-gel digested with trypsin, and subjected to MALDI-TOF/TOF analysis for assignment to protein families, as shown in Table 1.

Figure 2: Composition of *N. kaouthia* venom according to protein families, expressed as percentages of the total protein content. 3FTx: three-finger toxin; PLA₂: phospholipase A₂; NGF: nerve growth factor; OHA: Ohanin/vespryn; CRISP: Cysteine-rich secretory protein; CTL: C-type lectin/lectin-like; NUCL: nucleotidase; PDE: phosphodiesterase; MP: metalloproteinase; LAO: L-amino oxidase; CVF: Cobra venom factor; CTT: Cytidyltransferase; UNK: Unidentified proteins; NP: non-protein components. A division between cytotoxins (CYT) and neurotoxins (NTX) is given for the three-finger toxins.

Figure 3: Presence of selected nucleosides and FAD in *Naja kaouthia* venom shown by spiking crude venom with 10 µg of nucleosides (adenosine, inosine, guanosine) or FAD and separating the venom components by reverse-phase HPLC. If a the peak of a nucleoside coincides with the peak of a venom component, and if mass determination yielded the same mass for the venom component as calculated for the nucleoside, the venom component was

judged to consist of the corresponding nucleoside. *N. kaouthia* venom contains small amounts of adenosine, guanosine, and traces of inosine, but no FAD.

Figure 4: (A) Comparison of the phospholipase A₂ activity between the venoms of *Naja kaouthia* and *Micrurus nigrocinctus*. *N. kaouthia* displays some enzymatic activity, although significantly less than *M. nigrocinctus*. (B) Comparison of the proteolytic activity between the venoms of *N. kaouthia*, *M. nigrocinctus*, and *Bothrops asper*, evaluated on azocasein. *N. kaouthia* shows negligible proteinase activity.

Figure 5: Electrospray ionization-mass spectrometry analysis of *N. kaouthia* venom fractions 6 and 10 (see Fig.1 and Table 1). Samples were analyzed in enhanced multi-charge positive mode as described in Methods. Fraction 6 (Nk-6) presented a homogeneous multi-charged series (A) which deconvoluted to an isotope-averaged mass (M_{av}) of 7826.0 ± 0.8 Da (B). The multi-charged series of fraction 10 (Nk-10) (C) showed a main protein with M_{av} 7619.0 ± 1.3 Da and a less abundant protein of 7656.0 ± 0.7 Da (D), thus demonstrating difference between fractions 6 and 10.

Figure 6: ELISA-based immunoprofiling of human antibodies (**Donor 1 IgG:** protein A-purified IgG antibodies from Donor 1, **Donor 2:** protein A-purified IgG antibodies from Donor 2, **Human negative:** normal human serum from healthy volunteer, never bitten by a snake) to the fractions of *Naja kaouthia* venom separated by RP-HPLC (see Materials and Methods for details). Toxins with the highest Toxicity Score (4, 5, and 6) are marked with a skull symbol.

Table 1: Assignment of the RP-HPLC isolated fractions of *Naja kaouthia* venom to protein families by MALDI-TOF-TOF of selected peptide ions from in-gel trypsin-digested protein bands.

Peak	%	Mass (kDa) ▼	Peptide ion <i>m/z</i>	MS/MS-derived or N-terminal (Nt) sequence	Conf (%)	Sc	Protein family; ~ related protein *
1	0.2	-	-	1 non-peptidic	-	-	unknown
2	0.4	-	-	1 non-peptidic (Adenosine)	-	-	non-peptidic (Nucleosides)
3	0.5	-	-	1 non-peptidic (Guanosine)	-	-	non-peptidic (Nucleosides)
4	1.2	13	2945.2	1 XECHNBSSBTPTTGGCSGGETNCYK	99	25	3FTx three-finger toxin <i>Naja atra</i> , ~E2IU03
5a	0.3	67	1475.7	1 SXDXXBBWEEBS(R ^b)	99	11	Cytidyltransferase choline-phosphate cytidylyltransferase A isoform X2 <i>Python bivittatus</i> , ~XP_007440684
5b	0.4	54	-	-	-	-	unknown
5c	3.3	10	1728.7 1014.5 1316.6	1 XECHNBSSBAPTTK 1 BWWSDDR 1 VBPGVNXNCCR	99 99 99	15 8 11	3FTx cobrotoxin-c <i>Naja kaouthia</i> , ~P59276
			1453.6 2945.2	1 (N ^{db})GXEXNCCCTDR 1 XECHNBSSBTPTTGGCSGGETNCYK	99 99	12 28	three-finger toxin <i>Naja atra</i> , ~E2IU03
6a	13.5	13	1758.8	1 XECHNBSSBTPTTK	99	13	cobrotoxin-b <i>Naja kaouthia</i> , ~P59275
			1315.5 2241.9	1 TWCDAFCSXR 1 TGVDXBCCSTDNCNPFPTK	99 99	13 16	3FTx alpha-elaptotoxin-NK2a <i>Naja kaouthia</i> , ~P01391
6b	18.8	10	1547.7 2184.9 1315.5 2241.9	1 RVDXGCAATCPTVK 1 TGVDXBCCSTDNCNPFPTK 1 TWCDAFCSXR 1 TGVDXBCCSTDNCNPFPTK	99 99 99 99	13 13 14 24	3FTx alpha-elaptotoxin-NK2a <i>Naja kaouthia</i> , ~P01391
7	0.9	10	1315.5 2241.9	1 TWCDAFCSXR 1 TGVDXBCCSTDNCNPFPTK	99 99	13 21	3FTx alpha-elaptotoxin-NK2a <i>Naja kaouthia</i> , ~P01391
8	4.1	10	2187.9 1011.5 1629.6 2578.0 2391.0	1 SXFGVTTEDCPDGBNXCFK 1 WH(M ^{ox})XVPGR 1 XTCXNCPEMFCGK 1 GCADTCVPGBPYEMXECCSTDK 1 XTCXNCPEMFCG(K ^{ox})FBXCR	99 82.8 99 99 99	23 8 16 28 11	muscarinic toxin-like prot.2 <i>Naja kaouthia</i> , ~P82463 3FTx weak tryptophan-containing neurotoxin, <i>Naja kaouthia</i> , ~P82935

9a	0.3	10	1415.6 1363.6 2603.1 2260.9	1 1 1 1	CBNPDPSPGCR AXTMEGNBASWR EDHPVHNXGEHSVCDSVSAWVTK GXDSHWNSTCTETDTFXK	99 99 99 99	10 18 29 27	Nerve growth factor NGF <i>Naja kaouthia</i> , ~P61899
9b	0.4	10	962.4 1177.5 2186.8 2241.9 1315.5	1 1 1 1 1	BYFETK TXCYNHXTR TSEFTXCPDSWYFCYK TGVDXBCCSTDNCNPFTTR TWCDAFCSXR	99 99 99 99 98.8	11 7 17 11 10	NGF <i>Naja sputatrix</i> , ~Q5YF89 3FTx muscarinic toxin-like prot.3 <i>Naja kaouthia</i> , ~P82464 alpha-elaptotoxin-NK2a <i>Naja kaouthia</i> , ~P01391
10	10.2	10	1629.6 1315.5 2241.9	1 1 1	XTCXNCPMEFCGK TWCDAFCSXR TGVDXBCCSTDNCNPFTTR	99 99 99	14 13 15	weak neurotoxin NNAM2 <i>Naja atra</i> , ~Q9YGI4 3FTx alpha-elaptotoxin-NK2a <i>Naja kaouthia</i> , ~P01391
11a	4.9	15	1842.7	1	SWWDFADYGCCYCGR	99	10	Phospholipase A₂ acidic PLA ₂ - 2 <i>Naja sagittifera</i> , ~P60044
11b	2.0	10	1826.6 1669.5 1232.5 1697.6 1842.6 900.4	1 1 1 1 1 1	CCBVHDNCYNEAEK GDNDACAAGVCDCCR NMXXBCTVPINR TYSYECBSBGITXICK SWWDFADYGCCYCGR CWPLYEK	99 99 99 99 99 98.2	22 20 13 16 18 9	Phospholipase A₂ acidic PLA ₂ - 1 <i>Naja kaouthia</i> , ~P00596
12a	2.1	15	1842.6	1	SWWDFADYGCCYCGR	99	11	Phospholipase A₂ acidic PLA ₂ - 1 <i>Naja sputatrix</i> , ~Q91900
12b	2.0	10	1610.5 1842.6 1697.6 1157.5 1826.6 2356.0	1 1 1 1 1 1	GGNNACAAAACVDCDR SWWDFADYGCCYCGR TYSYECBSBGITXICK XSGCWPLYEK CCBVHDNCYNEAEK XAAXCFAGAPYNNNNNTNXDXK	99 99 99 99 99 99	20 19 14 13 20 22	Phospholipase A₂ acidic PLA ₂ - 1 <i>Naja atra</i> , ~P00598
13a	2.5	22	1842.6	1	SWWDFADYGCCYCGR	99	9	Phospholipase A₂ acidic PLA ₂ - D <i>Naja sputatrix</i> , ~Q91900
13b	5	10	2829.3 1087.4 1798.8	1 1 1	TCPAGBNXXCYBMFMVSNBTVPVBR YVCCNTDR NSXXV(K ⁴⁹)YVCCNTDR	99 99 99	14 10 15	3FTx cytotoxin 3 <i>Naja kaouthia</i> , ~P01446
			1842.6	1	SWWDFADYGCCYCGR	99	14	Phospholipase A₂

14a	1.3	25	1087.4	1	YVCCNTDR	91.6	7	acidic PLA ₂ - D <i>Naja sputatrix</i> , ~Q91900
								3FTx
								cytotoxin VC-1 <i>Naja oxiana</i> , ~Q9PS33
14b	1.1	10	2829.3	1	TCPAGBNXCYBMFMMSDXTXPVBR	99	10	3FTx
			1087.4	1	YVCCNTDR	99	12	cytotoxin 2 <i>Naja kaouthia</i> , ~P01445
			1798.8	1	NSXXV(K ^{eu})YVCCNTDR	99	15	
15	2.4	11	1412.6	1	MFMMSDXTXPVK	99	11	3FTx
			1798.8	1	NSXXV(K ^{eu})YVCCNTDR	99	15	cytotoxin VC-1 <i>Naja oxiana</i> , ~Q9PS33
			1568.7	1	MFMMSDXTXPVBR	99	14	
			1087.4	1	YVCCNTDR	99	10	
								3FTx
								cytotoxin 5V <i>Naja atra</i> , ~Q9W716
16a	5.0	21	1420.6	1	CHNTBXPPEXYK	99	16	
			1087.5	1	YVCCNTDR	man	man	3FTx
								three-finger toxin <i>Naja atra</i> , ~E2IU04
16b	3.7	10	2861.3	1	TCPAGBNXCYBMFMMSDXTXPVBR	99	11	3FTx
			1412.6	1	MFMMSDXTXPVK	99	11	three-finger toxin <i>Naja atra</i> , ~E2IU04
			1568.7	1	MFMMSDXTXPVBR	99	17	
			1798.7	1	NSXXV(K ^{eu})YVCCNTDR	99	11	
			1087.4	1	YVCCNTDR	99	10	
17a	0.5	12	2141.9	1	ADVTFDNSTAFESXVVSPDK	99	15	Ohanin/vespryn
			2270.0	1	ADVTFDNSTAFESXVVSPDBK	99	17	thaicobrin <i>Naja kaouthia</i> , ~P82885
			930.4	1	EWAVGXAGK	99	13	
			913.4	1	SPPGNWBK	99	10	
			1810.8	1	TVENVGVSBVAPDNPER	99	19	
			1495.6	1	FDGSPCVXGSPGFR	99	19	
17b	1.2	10	1771.8	1	SSXXV(K ^{eu})YVCCNTDR	99	14	3FTx
			2918.2	1	GCXDVCPSBSSXXVBVYVCCNTDRCN	83.4	7	cytotoxin 4N <i>Naja atra</i> , ~Q9W6W9
			1087.4	1	YVCCNTDR	99	11	
								3FTx
								cytotoxin 3a <i>Naja atra</i> , ~Q98959
18a	0.8	13	1798.8	1	NSXXV(K ^{eu})YVCCNTDR	99	16	
			1315.5	1	TWCDAFCSSXR	94.7	8	3FTx
								alpha-elapitoxin-Nk2a <i>Naja kaouthia</i> , ~P01391
18b	4.6	10	1087.4	1	YVCCNTDR	99	10	3FTx
			948.4	1	GCXDVCVK	99	10	cytotoxin 8 <i>Naja atra</i> , ~Q91124
			924.4	1	MFMVATPK	99	10	
			2918.3	1	GCXDVCPSBSSXXVBVYVCCNTDRCN	99	11	
			1771.8	1	SSXXV(K ^{eu})YVCCNTDR	99	14	
19	0.2	10	-	-	-	-	-	unknown

20a	0.1	57	-	-	-	-	unknown
20b	0.5	20	2102.9	1	XGCGENXFMSBPYAWSR	99	Cysteine-rich secretory protein
			1409.7	1	VXBSWYDENBK	99	natrin-2 <i>Naja atra</i> , ~Q7ZZN8
20c	0.1	12	1810.9	1	TVENVGVBVAPDNPER	99	Ohanin/vespryn
			2270.0	1	ADVTEDSNTAFESXVSPDBK	99	thacobirin <i>Naja kaouthia</i> , ~P82885
			1495.7	1	FDGSPCVXGSPGFR	99	
20d	0.1	10	-	-	-	-	unknown
21	0.3	11	1296.6	1	BYXWEWTDNR	99	C-type lectin/lectin-like
			1168.5	1	YXWEWTDNR	99	LP-Pse-6 CTL <i>Pseudonaja modesta</i> , ~R4G314
22a	1.1	21	2013.9	1	VXEGXBCGESXYMISSNAR	99	Cysteine-rich secretory protein
			1434.7	1	ZBEXVDXHNLSXR	99	natrin-1 <i>Naja atra</i> , ~Q7T1K6
			1553.6	1	MEWYPEAASNAER	99	
			3174.5	1	NFVYGVGANPPGSVTGHYTBXVWYBYR	99	
			1870.8	1	TWTEXXHXWHDEYK	99	
			1258.4	1	SNCPASCFCR	99	
			1784.8	1	WANTCSXNHSPDNXR	99	
22b	0.1	15	1553.6	1	MEWYPEAASNAER	99	Cysteine-rich secretory protein
			1784.7	1	WANTCSXNHSPDNXR	99	natrin-1 <i>Naja atra</i> , ~Q7T1K6
			1870.8	1	TWTEXXHXWHDEYK	99	
			2013.9	1	VXEGXBCGESXYMISSNAR	99	
22c	0.1	12	1168.5	1	YXWEWTDNR	99	C-type lectin/lectin-like
			1296.6	1	BYXWEWTDNR	97.6	LP-Pse-6 CTL <i>Pseudonaja modesta</i> , ~R4G314
22d	0.1	10	1168.5	1	YXWEWTDNR	98.5	C-type lectin/lectin-like
						7	LP-Pse-6 CTL <i>Pseudonaja modesta</i> , ~R4G314
23a	0.1	56	1470.8	1	XPVYSAYVYNPGK	99	Nucleotidase
			1695.9	1	SSTFTXTNXVPBFXK	99	endonuclease domain-containing 1 prot <i>Micrurus</i>
			1635.8	1	GHXNPNGHBPDPYSAK	99	<i>fuhrus</i> , ~tr[U3FCT9
			2024.0	1	EVVDSFBDHCPBFXR	99	
23b	0.2	26	2080.9	1	GEVVDSFBDHCPBFXR	99	Nucleotidase
			2023.8	1	EVVDSFBDHCPBFXR	99	endonuclease domain-containing 1 prot, <i>Micrurus</i>
			1695.8	1	SSTFTXTNXVPBFXK	96.9	<i>fuhrus</i> , ~U3FCT9
24a	0.1	250	1430.8	1	YXEFVYXVDNR	99	Metalloproteinase
						10	kaouthagin <i>Naja kaouthia</i> , P82942
24b	0.2	66	2689.1	1	HDGDXPEXCTGBSAECPDTSXBR	99	Metalloproteinase
			1099.6	1	DYBEYXXR	99	kaouthagin <i>Naja kaouthia</i> , P82942
			1471.7	1	TAPAFBSSCSXR	99	

24c	0.2	57	1430.8	1	YXEFYVXVDNR	99	15	
			1099.5	1	DYBEYXXR	99	10	Metalloproteinase
			2689.1	1	HDCDXPEXCTGBSAECPDTSXBR	99	13	kaouthagin <i>Naja kaouthia</i> , P82942
			1471.7	1	TAPAFBSSCSXR	99	14	
			1430.7	1	YXEFYVXVDNR	99	15	
24d	0.7	40	2354.1	1	CPTXTNBCXAXXGPHFTVSPK	99	25	Metalloproteinase
			1471.6	1	TAPAFBSSCSXR	99	17	kaouthagin <i>Naja kaouthia</i> , ~P82942
			1912.6	1	(N ¹⁴⁶)GHPCBNNNBGYCY(N ¹⁴⁹)GK	99	23	
			1012.4	1	GCFDXXNMR	99	11	
			1430.7	1	YXEFYVXVDNR	99	13	
			2689.0	1	HDCDXPEXCTGBSAECPDTSXBR	99	25	
			1099.5	1	DYBEYXXR	99	12	
25a	0.1	139	1434.7	1	DFYTFDSEAXVK	99	8	Phosphodiesterase
			1244.7	1	SP(N ¹⁴⁶)NXWVEER	99	9	phosphodiesterase 1 <i>Micrurus fulvius</i> , ~U3FAB3
			2106.1	1	RPDFTXYYXEEPDTTGHK	99	11	
25b	0.3	108	1434.7	1	DFYTFDSEAXVK	99	16	Phosphodiesterase
			1244.7	1	SP(N ¹⁴⁶)NXWVEER	99	15	phosphodiesterase 1 <i>Micrurus fulvius</i> , ~U3FAB3
			2106.1	1	RPDFTXYYXEEPDTTGHK	99	20	
			1634.7	1	YCSGGTHGYDNEFK	99	14	
			1518.9	1	XWNVYHSTXXPK	99	11	
			1355.7	1	AATYFWPGSEVK	99	9	
25c	0.1	61	1449.8	1	VVSXNVXCTEGR	99	8	5'-nucleotidase
			2421.3	1	FHECNXGNXXCDVYYNNXR	99	9	ecto-5'-nucleotidase 1 <i>Micrurus fulvius</i> , ~U3FYYP9
			1719.0	1	ETPVXSNPGPYXEFR	99	10	
			1459.8	1	YXGYXNVXFDDK	93,5	7	
26a	0.1	250	1339.7	1	EGWYVNMGPMPR	99	9	L-amino acid oxidase
			1718.9	1	TFVTADYVXYV/CSTSR	99	15	L-amino-acid oxidase <i>Naja atra</i> , ~A8QL58
			2517.2	1	SPXEECFBBNDYEEFXEXAR	99	20	L-amino-acid oxidase <i>Naja oxiama</i> , ~P0D191
26b	0.1	70	2030.0	1	FFXXDGNENFHVXSXTAR	99	12	Cobra venom factor
			1386.7	1	BXDXFVHDPPR	99	10	CVF <i>Naja kaouthia</i> , Q91132
			1875.1	1	XXXXNXPXNABSPXTVR	99	13	
26c	0.3	55	2673.3	1	RSPXEECFBBNDYEEFXEXAR	99	8	L-amino acid oxidase
			2922.5	1	FGXBXNEFFBENENAWYYXNNXR	99	10	L-amino-acid oxidase <i>Naja oxiama</i> , ~P0D191
			2517.2	1	SPXEECFBBNDYEEFXEXAR	99	17	
27	0.3	46	1603.8	1	VYEMVNAIXNTMYR	99	11	Metalloproteinase
			1235.7	1	VTXDXFGEWR	99	12	mocarhagin, <i>Naja mossambica</i> , ~Q10749
28	0.9	44	2713.2	1	DDCDXPEXCTGBSAECPDTVFBR	99	11	Metalloproteinase

1207.6	1	ATXDXFGIEWR	99	10	cobrin <i>Naja kaouthia</i> , ~Q9PVK7
1807.9	1	YXEFY(M ^{db})VVDNXXMYR	99	12	
2983.4	1	AABDDCDXPEXCTGBSAECPTDVFBK	99	10	

* Cysteine residues are carbamidomethylated, unless underlined. Confidence (Conf) and Score (Sc) values are calculated by the Paragon algorithm of ProteinPilot[®] (ABSciex) ▼ : estimated mass by SDS-PAGE under reducing conditions, in kDa. V : observed isotope-averaged masses as determined by nESI-MS of selected RP-HPLC peaks, in Da. X: Leu/Ile; B: Lys/Gln; Z: pyroglutamate (2-oxo-pyrrolidone carboxylic acid). Possible, although unconfirmed/ambiguous amino acid modifications suggested by the automated identification software are shown in parentheses, with the following abbreviations: ^{ox}: oxidized; ^{dn}: deamidated; ^{ca}: carbamyl; ^{am}: amide; ^{nc}: Na cation; ^{dl}: ammonia loss; ^{dc}: delta ^{H2C2}, man: manually interpreted MS/MS spectrum.

Table 2: Median lethal doses (LD₅₀s) of *Naja kaouthia* venom and the RP-HPLC isolated fractions

Peak	%	Protein family	LD ₅₀ (95% C.I.)	Reported LD ₅₀ (mg/kg)	Toxicity Score ¹ % / LD ₅₀ (kg/mg)
Whole venom					
4	1.2	3FTx three-finger toxin <i>Naja atra</i> , ~E2IU03	0.24 (0.12-0.41)	0.115*	423.2
5	4.0 (3:4:33 mix ²)	Cytidylyltransferase (7.5%) choline-phosphate cytidylyltransferase A isoform X2 <i>Python bivittatus</i> , ~XP_007440684	0.07 (0.001-0.19)	80 (cobrotoxin- c), 400 (cobrotoxin-b) [Meng et al., 2002]	41.3
		Unknown (10%)			
		3FTx (82.5%) cobrotoxin-c <i>Naja kaouthia</i> , ~P59276 three-finger toxin <i>Naja atra</i> , ~E2IU03 cobrotoxin-b <i>Naja kaouthia</i> , ~P59275			
6	32.3	3FTx alpha-elapitoxin-NK2a <i>Naja kaouthia</i> , ~P01391	0.10 (0.02-0.18)	0.1 [Karlsson, 1973]	326
7	0.9	3FTx alpha-elapitoxin-NK2a <i>Naja kaouthia</i> , ~P01391 muscarinic toxin-like prot.2 <i>Naja kaouthia</i> , ~P82463	0.36 (0.17-0.72)		2.5
8	8.0	3FTx weak tryptophan-containing neurotoxin, <i>Naja kaouthia</i> , ~P82935	2		4
9	0.7 (3:4 mix ²)	Nerve growth factor (43%) NGF <i>Naja kaouthia</i> , ~P61899 NGF <i>Naja sputatrix</i> , ~Q5YF89 3FTx (57%) muscarinic toxin-like prot.3 <i>Naja kaouthia</i> , ~P82464 alpha-elapitoxin-NK2a <i>Naja kaouthia</i> , ~P01391 weak neurotoxin NNAM2 <i>Naja atra</i> , ~Q9YGI4	>1		<1

10	10.2	3FTx alpha-elapitoxin-NK2a <i>Naja kaouthia</i> , ~P01391	>10	<1
11	6.9 (49:20 mix ²)	Phospholipase A₂ acidic PLA ₂ - 2 <i>Naja sagittifera</i> , ~P60044 (71%) acidic PLA ₂ - 1 <i>Naja kaouthia</i> , ~P00596 (29%)	5.38 (2.01-55.94)	10 [Chuman et al., 2000] 1.3
12	4.1 (51:49 mix ²)	Phospholipase A₂ acidic PLA ₂ - 1 <i>Naja sputatrix</i> , ~Q91900 (51%) acidic PLA ₂ - 1 <i>Naja atra</i> , ~P00598 (49%)	3.74 (2.17-8.75)	1.1
13	7.5 (1:2 mix ²)	Phospholipase A₂ (33%) acidic PLA ₂ - D <i>Naja sputatrix</i> , ~Q91900 3FTx (66%) cytotoxin 3 <i>Naja kaouthia</i> , ~P01446	0.9 (0.09-1.29)	8.3
14	2.4 (27:23 mix ²)	3FTx cytotoxin VC-1 <i>Naja oxiata</i> , ~Q9PS33 (54%) cytotoxin 2 <i>Naja kaouthia</i> , ~P01445 (46%)	1.60 (1.21-2.28)	1.2 (cytotoxin 2) [Joubert and Taljaard, 1980b] 1.57
15	2.4	3FTx cytotoxin VC-1 <i>Naja oxiata</i> , ~Q9PS33	1-2.5	<2.5
16	8.7	3FTx three-finger toxin <i>Naja atra</i> , ~E21U04	0.75 (0.30-1.66)	11.6
17	1.7 (5:12 mix ²)	Ohain/vespryn (29%) thaicobrin <i>Naja kaouthia</i> , ~P82885 3FTx (71%) cytotoxin 4N <i>Naja atra</i> , ~Q9W6W9 cytotoxin 3a <i>Naja atra</i> , ~Q98959	1.36 (0.62-2.03)	1.25
18	5.4 (4:23 mix ²)	3FTx (15%) alpha-elapitoxin-NK2a <i>Naja kaouthia</i> , ~P01391 3FTx (85%) cytotoxin 8 <i>Naja atra</i> , ~Q91124	1.00 (0.43-2.93)	5.4
19	0.2	Unknown	>0.25	<1

		Unknown (25%)		
20	0.8 (2:5:1 mix ²)	Cysteine-rich secretory protein (62.5%) natriin-2 <i>Naja atra</i> , ~Q7ZZN8 Ohanin/vespryn (12.5%) thaicobrin <i>Naja kaouthia</i> , ~P82885	>1	<1
21	0.3	C-type lectin/lectin-like LP-Pse-6 CTL <i>Pseudonaja modesta</i> , ~R4G314	>0.5	<1
22	1.4 (6:1 mix ²)	Cysteine-rich secretory protein (86%) natriin-1 <i>Naja atra</i> , ~Q7TIK6 C-type lectin/lectin-like (14%) LP-Pse-6 CTL <i>Pseudonaja modesta</i> , ~R4G314	>1.5	<1
23	0.3	Nucleotidase endonuclease domain-containing 1 prot <i>Micrurus fulvius</i> , ~tr U3FCT9		N.t.
24	1.2	Metalloproteinase kaouthiagin <i>Naja kaouthia</i> , P82942		N.t.
25	0.5 (4:1 mix ²)	Phosphodiesterase (80%) phosphodiesterase 1 <i>Micrurus fulvius</i> , ~U3FAB3 5'-nucleotidase (20%) ecto-5'-nucleotidase 1 <i>Micrurus fulvius</i> , ~U3FYYP9	>0.25	<2
26	0.5 (1:1:3 mix ²)	L-aminic acid oxidase (20%) L-aminic acid oxidase <i>Naja atra</i> , ~A8QL58 L-aminic acid oxidase <i>Naja oxiana</i> , ~P0D191 Cobra venom factor (20%) CVF <i>Naja kaouthia</i> , Q91132 L-aminic acid oxidase (60%) L-aminic acid oxidase <i>Naja oxiana</i> , ~P0D191		N.t.
27	0.3	Metalloproteinase mocarhagin, <i>Naja mossambica</i> , ~Q10749		N.t.
28	0.9	Metalloproteinase cobrin <i>Naja kaouthia</i> , ~Q9PVK7		N.t.

*<http://snakedatabase.org/pages/LD50.php#legendAndDefinitions>

¹ Toxicity Score was defined as the ratio of protein fraction abundance (%) in the venom divided by its estimated median lethal dose (LD₅₀) for CD-1 mice by i.v. injection.

² Mix indicates that the fraction did not contain a pure, isolated toxin, but instead a mixture of 2-4 different toxins in variable ratios indicated in the table.

N.t. : not tested

Figure 1
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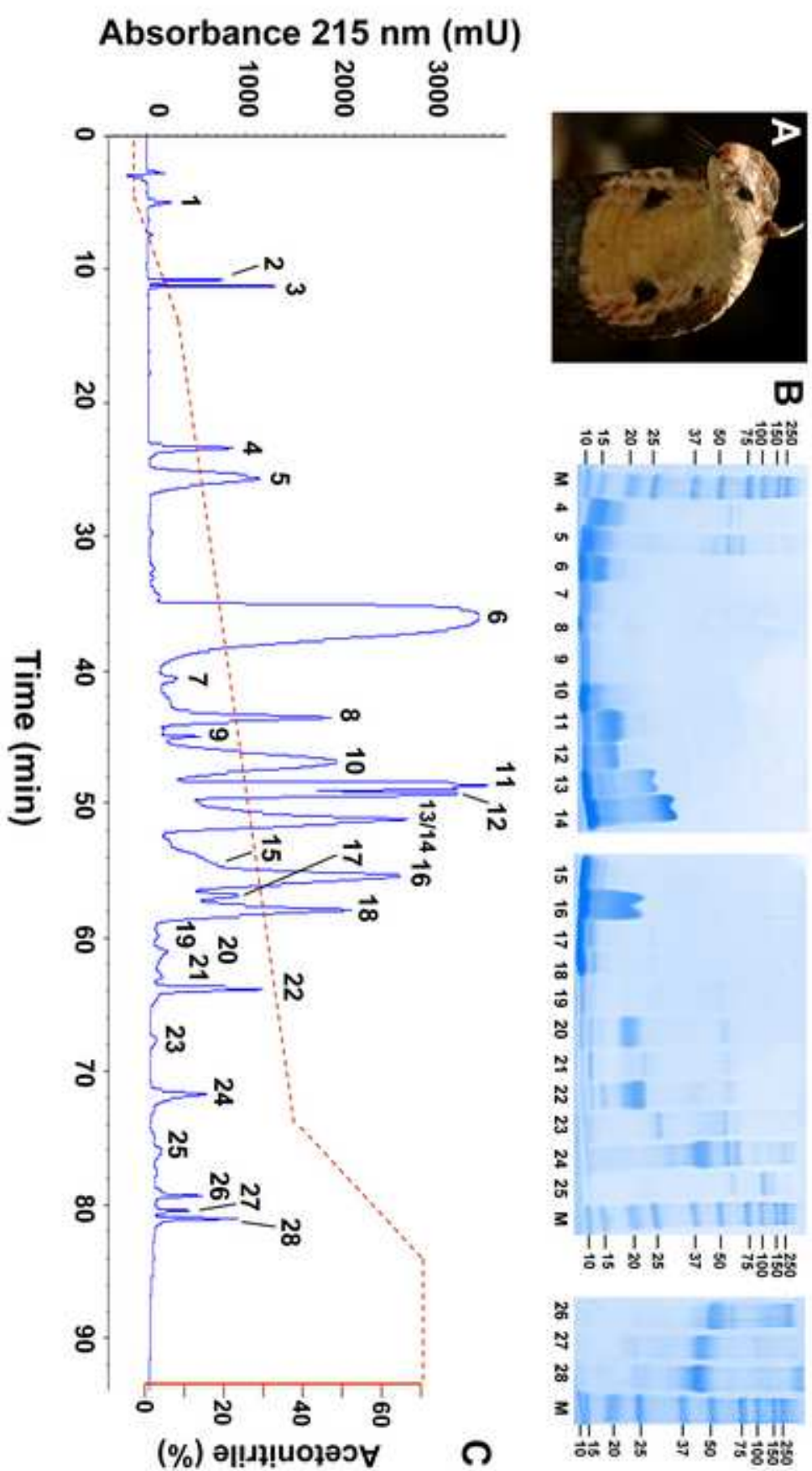


Figure 2
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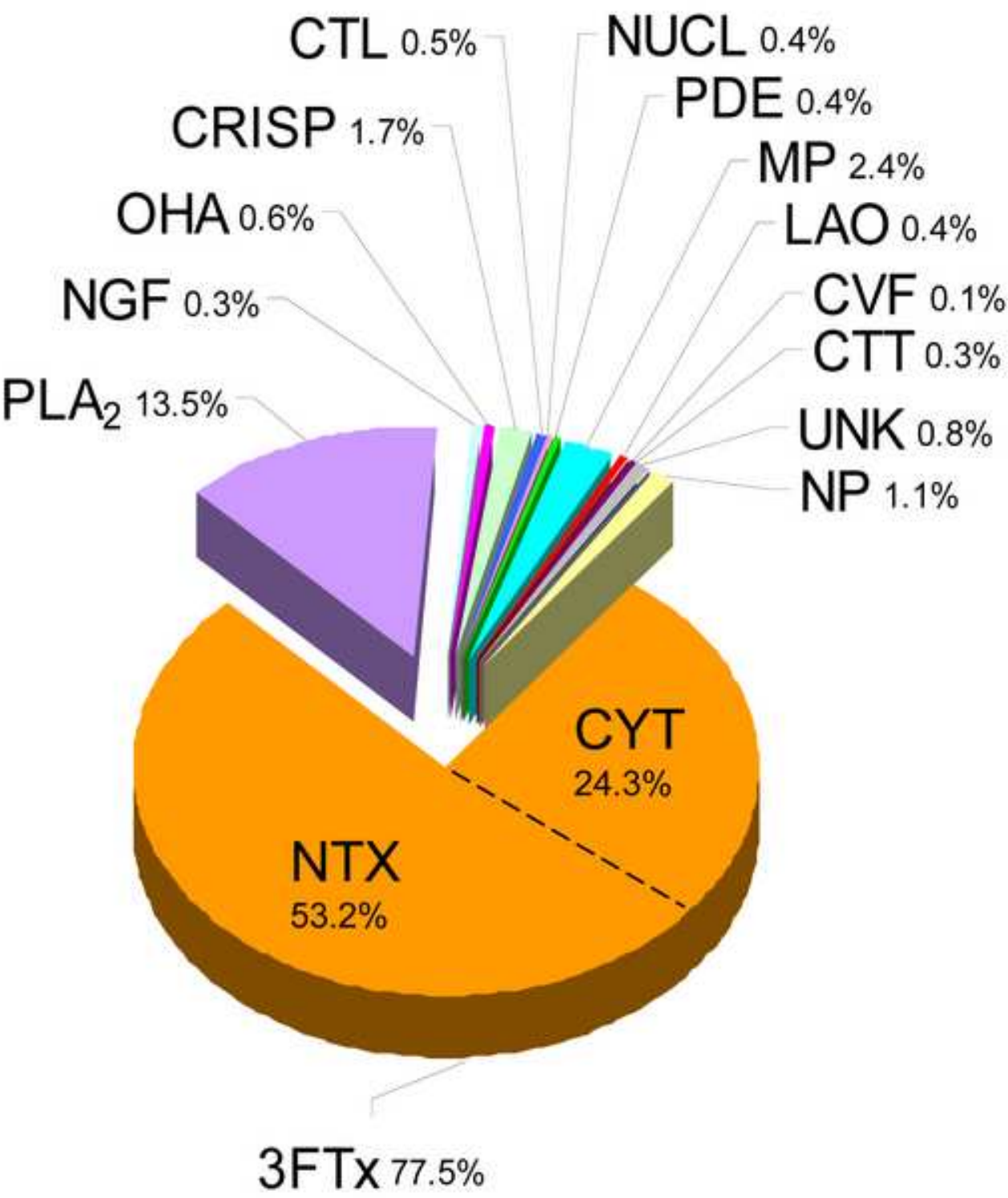


Figure 3
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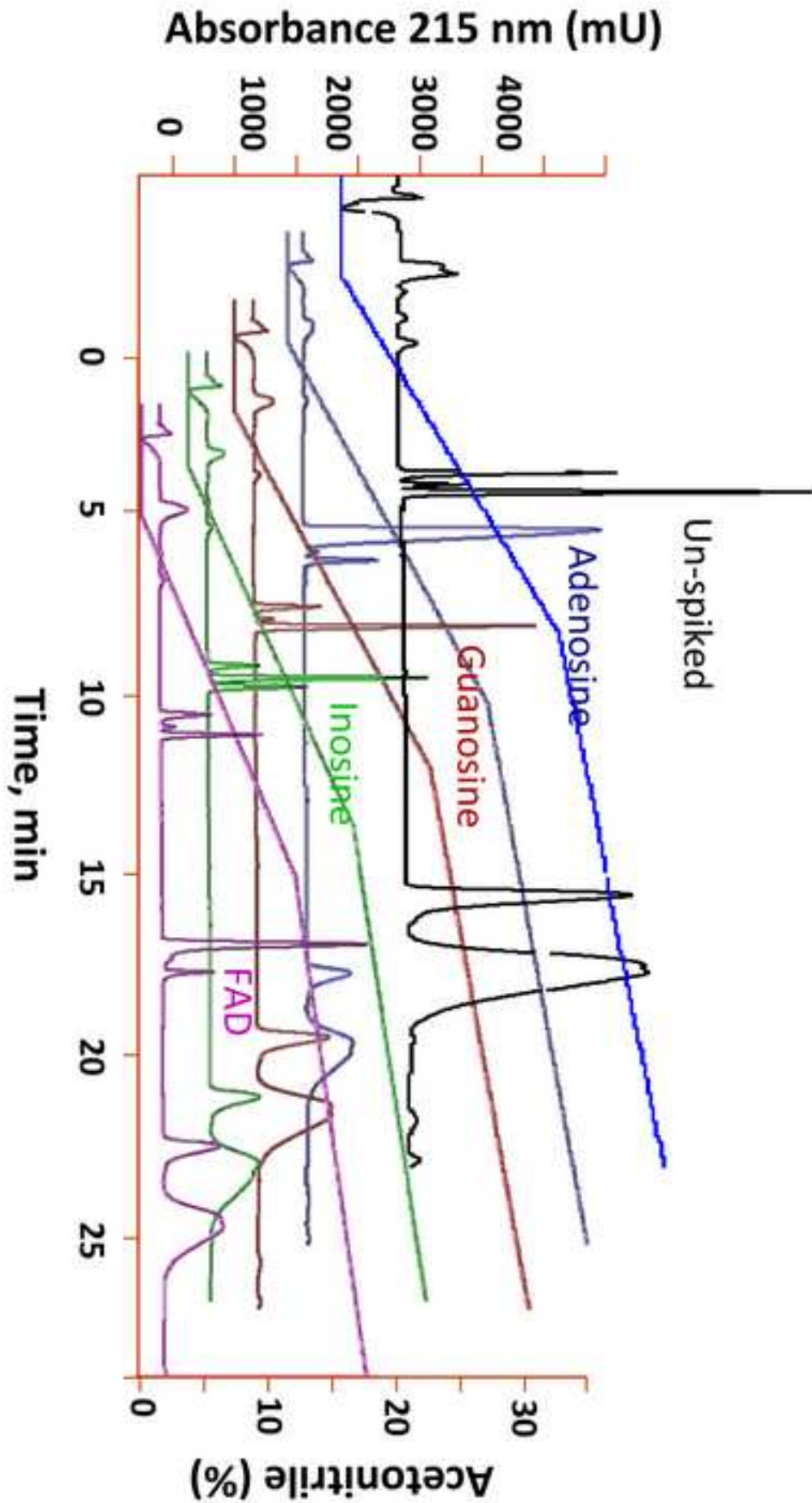


Figure 4
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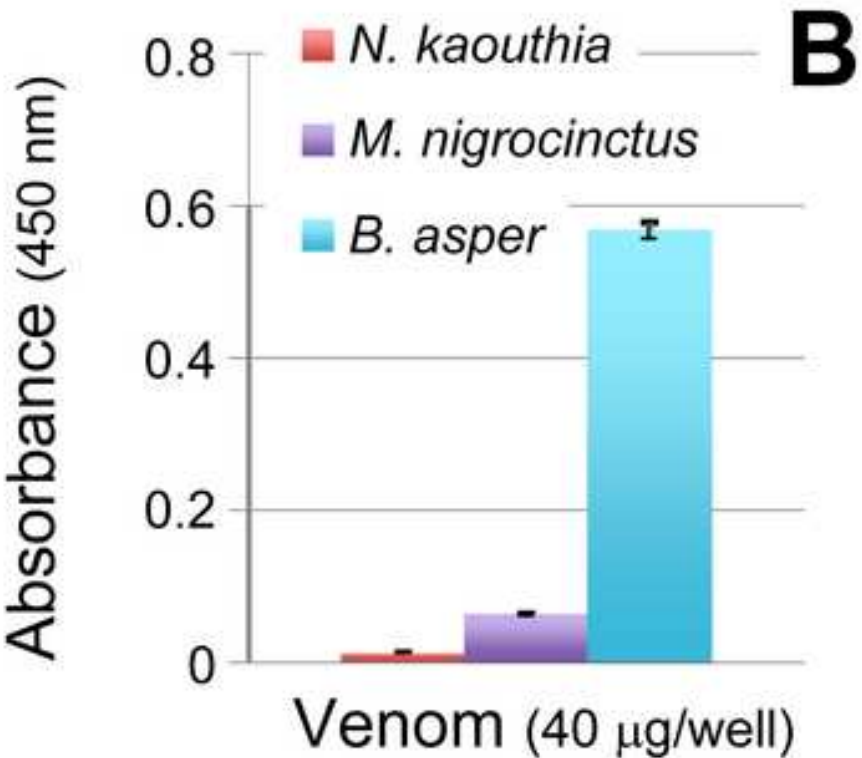
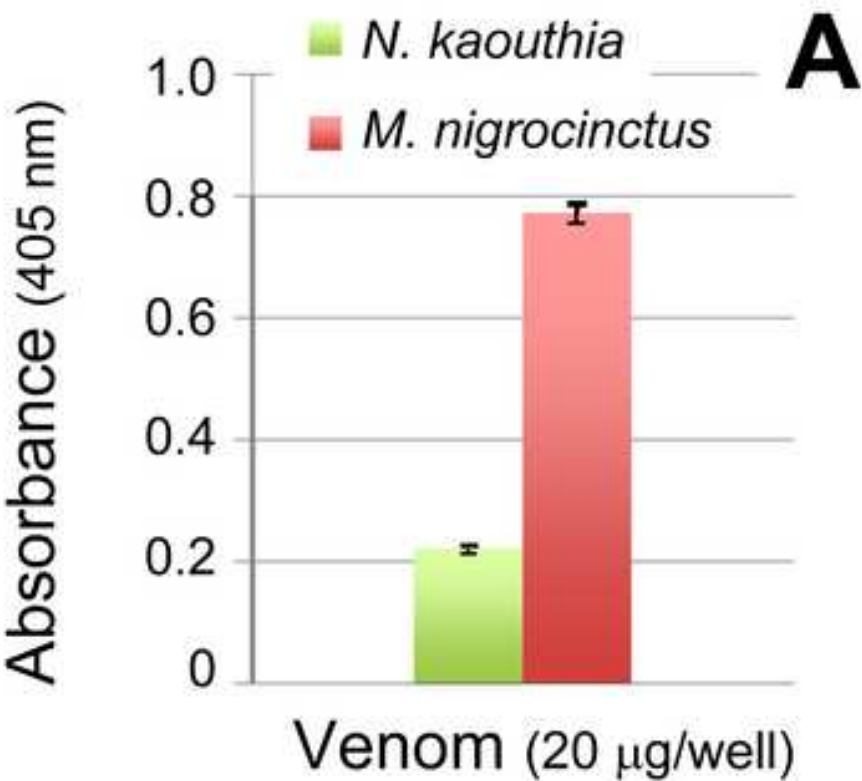


Figure 5
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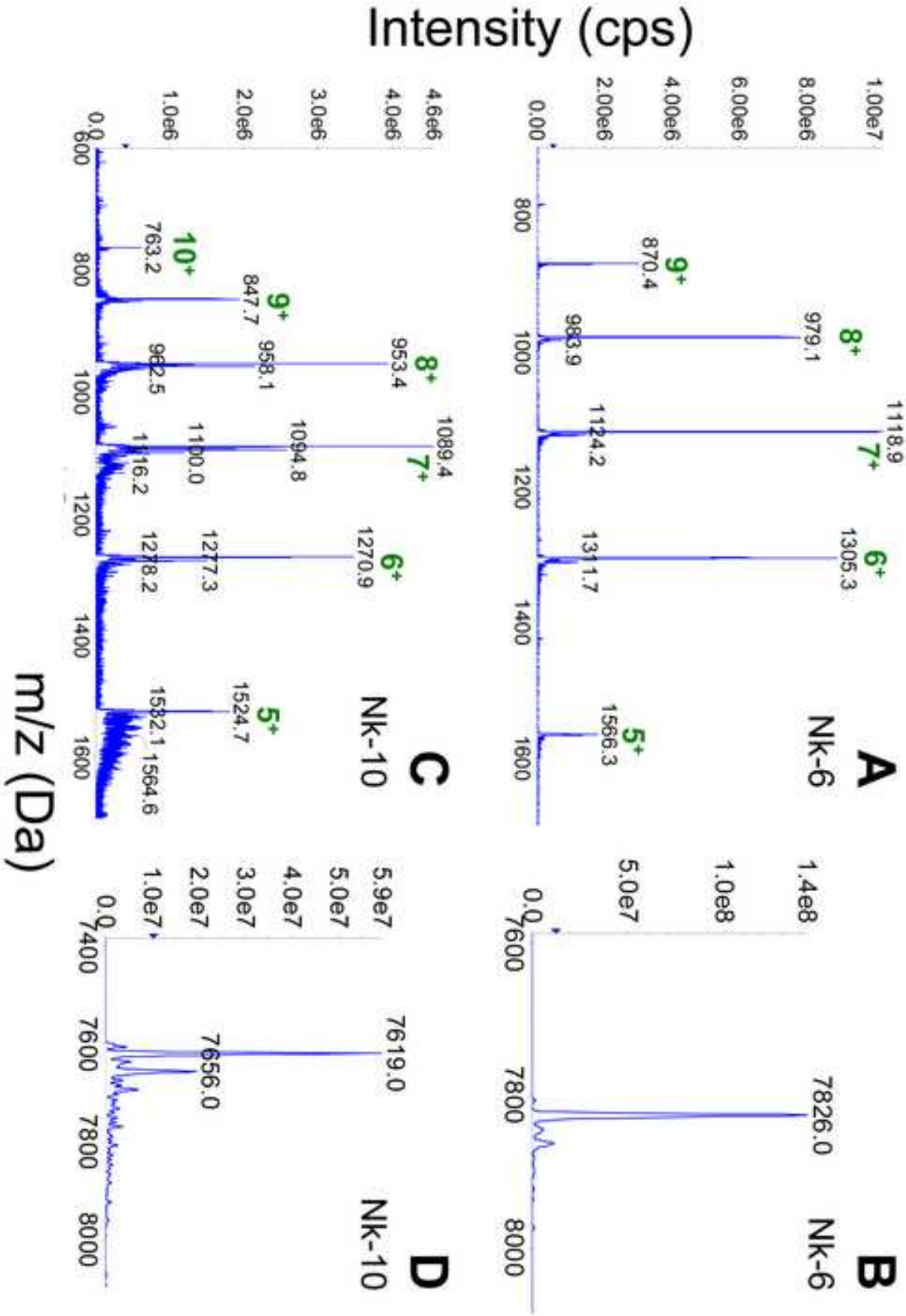


Figure 6
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